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Sexual functionality of *Leptopilina clavipes* (Hymenoptera: Figitidae) after reversing *Wolbachia*-induced parthenogenesis

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Leptopilina clavipes;
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sexual function decay;
thelytoky;
Wolbachia.

Abstract

Females infected with parthenogenesis-inducing *Wolbachia* bacteria can be cured from their infection by antibiotic treatment, resulting in male production. In most cases, however, these males are either sexually not fully functional, or infected females have lost the ability to reproduce sexually. We studied the decay of sexual function in males and females of the parasitoid *Leptopilina clavipes*. In western Europe, infected and uninfected populations occur allopatrically, allowing for an investigation of both male and female sexual function. This was made by comparing females and males induced from different parthenogenetic populations with those from naturally occurring uninfected populations. Our results indicate that although males show a decay of sexual function, they are still able to fertilize uninfected females. Infected females, however, do not fertilize their eggs after mating with males from uninfected populations. The absence of genomic incompatibilities suggests that these effects are due to the difference in mode of reproduction.

Introduction

Wolbachia bacteria are cytoplasmic endosymbionts (α -proteobacteria) that infect a wide range of arthropod and nematode hosts. They are maternally inherited and enhance their transmission by altering the reproductive system of their host in various ways, such as cytoplasmic incompatibility, male killing, feminization and parthenogenesis induction (PI) (Stouthamer *et al.*, 1999). All these phenomena lead to an increase in infected females.

Parthenogenesis-inducing *Wolbachia* are mainly found in the arthropod group Hymenoptera (Huigens & Stouthamer, 2003), and sporadically in other groups, such as Coleoptera (Werren *et al.*, 1995), Thysanoptera (Arakaki *et al.*, 2001) and in mites of the genus *Bryobia*

(Weeks & Breeuwer, 2001). PI *Wolbachia* are restricted to hosts with haplodiploid modes of reproduction (Huigens & Stouthamer, 2003) in which males are haploid and females are diploid. The most common mode of haplodiploid reproduction is arrhenotoky, where males develop from unfertilized eggs and females from fertilized eggs (White, 1973; Luck *et al.*, 1993). Females infected by PI *Wolbachia* produce all-female offspring (thelytoky) through gamete duplication (Stouthamer & Kazmer, 1994; Gottlieb *et al.*, 2002; Pannebakker *et al.*, 2004b).

Parthenogenesis-inducing *Wolbachia*-infected females can be cured from their infection by high temperature or antibiotic treatment (Stouthamer *et al.*, 1990a), which results in male production. In most cases however, either these males are sexually not fully functional (e.g. Zchori-Fein *et al.*, 1992, 1995; Gottlieb & Zchori-Fein, 2001), or the cured females have lost the ability to reproduce sexually (Pijls *et al.*, 1996; Arakaki *et al.*, 2000). Only in several *Trichogramma* species did the removal of PI *Wolbachia* results in the production of arrhenotokously reproducing lines (Stouthamer *et al.*, 1990a,b).

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In parthenogenetic populations, genes involved in sexual reproduction are not actively maintained by selection, and random mutations in those genes are thus not removed by selection (Muller, 1949; Carson *et al.*, 1982). Mutations can accumulate in these genes or, by means of antagonistic pleiotropy, even be actively selected for if they improve the parthenogenetic performance of females (Pijls *et al.*, 1996; Werren, 1998). The decay of sexual function in PI *Wolbachia*-infected systems has been studied extensively in species where infection has gone to fixation (e.g. Zchori-Fein *et al.*, 1992, 1995; De Barro & Hart, 2001; Gottlieb & Zchori-Fein, 2001; Weeks & Breeuwer, 2001). However, the decay of sexual function in both males and females can only be studied in species where infection status is polymorphic.

Here we study the decay of sexual function in males and females of *Leptopilina clavipes* (Hartig) (Hymenoptera: Figitidae), a parasitoid wasp of fungi-breeding *Drosophila* larvae that occurs in western Europe (Nordlander, 1980). In north-western Europe, populations of *L. clavipes* are thelytokous (Driessen *et al.*, 1990; Pannebakker *et al.*, 2004c), which is *Wolbachia* induced (Werren *et al.*, 1995; Schidlo *et al.*, 2002). Recently, uninfected arrhenotokous individuals were found in northern Spain (Pannebakker *et al.*, 2004c). In this study, we compare the sexual function of antibiotic curing-induced (ACI) males and *Wolbachia*-infected females from different thelytokous populations with that of material from natural arrhenotokous populations. We make the comparison at different levels: (i) male courtship behaviour (we include a description of *L. clavipes* courtship behaviour), (ii) male fertilization capacity, and (iii) sperm use by *Wolbachia*-infected females. In addition, we tested for the presence of genomic incompatibilities between the two modes of reproduction. Our results indicate that males show a decay of sexual function, but are still able to fertilize arrhenotokous females. Infected females however, do not use the sperm they receive after mating with arrhenotokous males. Genomic incompatibilities were absent between the two modes of reproduction and hence do not provide an explanation for our results.

Material and methods

Insect cultures

Leptopilina clavipes stocks

Twelve *L. clavipes* lines were used in the different experiments. The infected thelytokous lines BBH-NL00, DB23/9-NL99, DBK-NL00, GBW-NL00, HOD-NL00, KBH-NL00, VOSB-NL00 originated from the Netherlands, NEUVIC-F01 and RENNES-F01 originated from France. The uninfected arrhenotokous lines CCAP-E00, DC-E00 and Moll1-E00 originated from Spain. Collection details can be found in Pannebakker *et al.*, 2004c. Cultures were maintained in the laboratory on *Drosophila phalerata* larvae at 20 °C, L : D = 16 : 8 and 65% relative

humidity (RH). *Drosophila phalerata* were reared on a medium containing mushroom (*Agaricus bisporus*), water, dry yeast (*Saccharomyces cerevisiae* Hansen) and agar. The fungicides nipagin and propionic acid were added to prevent moulding of the medium.

Curing experiments and crosses were carried out using *D. subobscura* as a host. This species was reared on a patch of live bakers yeast (*S. cerevisiae*) suspension on a medium of water, dry yeast (*S. cerevisiae*) and agar at 20 °C, L : D = 16 : 8 and 65% RH.

Antibiotic treatment

To induce male offspring from the thelytokous line, infected females were cured from their *Wolbachia* infection using antibiotics applied in the honey (0.5% rifampicin) and in the host medium (0.2% rifampicin) as described by (Schidlo *et al.*, 2002). Curing was done using *D. subobscura* as a host on a live bakers yeast patch on agar at 25 °C, L : D = 16 : 8 and 65% RH.

Crossing experiments

Courtship behaviour

The courtship behaviour of ACI males from eight different thelytokous lines (BBH-NL00, DB17/9-NL99, DBK-NL00, GBW-NL00, HOD-NL00, KBH-NL00, NEUVIC-F01 and VOSB-NL00) was compared with that of arrhenotokous males (DC-E00). One virgin female (DC-E00) was placed in a glass mating arena (9 mm high × 32 mm in diameter) closed off with a thin glass plate. A single virgin male was introduced and the couple was observed under a dissecting microscope at 10× magnification. If no copulation attempt occurred within the first 15 min after the introduction of the male, a 'no copulation event' was scored. If copulation began within 15 min, the couple was observed until the mating stopped. Successful insemination following copulation was confirmed by allowing each mated female to oviposit on a patch with approximately 110 *D. subobscura* larvae for 24 h, and scoring the sex of the offspring. Age of the mating pairs was not strictly controlled for; mean age ± SD (days) males: 3.05 ± 3.46, females: 2.74 ± 3.33.

Observations were made at an ambient temperature of 20 °C and 65% RH, from mid-July to late September 2001. The male courtship behaviour was recorded using specialized computer software (Observer 3.0; Noldus Information Technology, Wageningen, The Netherlands, 1993). Descriptive statistics were calculated on the number of, and duration of the behavioural elements in courtship.

Behavioural data were analysed by fitting generalized linear models (GLM) with the appropriate error structures and link functions (i.e. transition probability data: binomial error structure and a logit link; latencies and durations: Gaussian error structure and identity link; count data: Poisson distribution and a log link). Moderate levels of overdispersion in the binomial and Poisson GLMs were corrected for by rescaling the deviance by the

heterogeneity factor (HF), the ratio of the residual deviance to the degrees of freedom (McCullagh & Nelder, 1989). For $HF > 3$ we used standard ANOVA after the appropriate transformation. Data were analysed using R statistical software (Ihaka & Gentleman, 1996, version 1.71). Model selection was made by comparing the Akaike Information Criterion (AIC) of the initial model with the AIC after excluding the line effect from the model. An *F*-test was used to determine the significance of the line effect. If significant line effects were observed for a behavioural element, the effects of the individual lines were compared using *F*-tests.

Sex ratio

Separate series of matings were performed to compare the sex ratio produced by ACI males (DB23/9-NL99, DBK-NL00, GBW-NL00, KBH-NL00, NEUVIC-F01, RENNES-F01 and VOSB-NL00) with that of natural arrhenotokous males (CCAP-E00 and DC-E00) when mating to virgin sexual females from a reference line (DC-E00). Matings were observed as described in the courtship behaviour assay. The experiments were carried out from late April to May 2003.

When a successful mating was observed, as defined by genital contact for over 30 s, the female was allowed to oviposit on a patch of approximately 130 *D. subobscura* larvae for 48 h. After the first oviposition period, the female was transferred to a new patch of approximately 130 *D. subobscura* larvae for 48 h after which the female and male were stored at -80°C for further genetic analysis. The larvae were incubated at 25°C and 65% RH, L : D = 16 : 8 and allowed to pupate. After pupation, the pupae were washed out of the medium and transferred to a new vial. Numbers of emerging males and females were recorded for a period of 4 weeks, during which most parasitoids eclosed. The noneclosed pupae were then opened and the sex of the parasitoids was recorded.

Sex ratio data are usually binomially distributed and are, therefore, best analysed using GLMs (Wilson & Hardy, 2002). We fitted a generalized linear mixed model to the data, using SAS/STAT software (SAS Institute, Cary, NC, USA) and a specific macro (GLIMMIX) as described in Littell *et al.* (1996). The proportion of males in a clutch was modelled as a binomial random variable, and regression models were coupled to this probability using the logit link function. Matings involving the same combination of lines were treated as repeated measures. Model selection occurred by first fitting an elaborate model. This was then simplified on the basis of likelihood ratio tests excluding factors for which the confidence intervals (CIs) overlapped the value zero. The initial model included brood size, arrhenotokous/thelytokous, first/second clutch and age of female and male as covariates, female and male as random effects, and allowed for a correlation between sex ratios in first and second clutches of the same mating pair.

Sperm utilization in infected females

To test whether infected females use the sperm of uninfected males to produce hybrid offspring, infected females were crossed to uninfected males (KBH-NL00 \times DC-E00, BBH-NL00 \times Moll1-E00, HOD-NL00 \times Moll1-E00). The parents and F_1 offspring were frozen (-80°C) for DNA analysis. Using amplified fragment length polymorphism (AFLP; Vos *et al.*, 1995) markers, the F_1 offspring were checked for the presence of paternal alleles. DNA isolation methods and AFLP procedures were as described in Pannebakker *et al.* (2004a), using the primer combination *Mse*-CA/*Eco*-ACA.

Recovery of parental alleles

To determine the existence of genomic incompatibilities between the two modes of reproduction, the recovery rate of alleles specific to each mode was examined in hybrid offspring. A cross was made between an ACI male from KBH-NL00 and a female from the arrhenotokous line DC-E00 under the conditions outlined above. Six female F_1 offspring were allowed to reproduce as virgins, resulting in 72 F_2 recombinant males. The parents and F_2 offspring were frozen (-80°C) for DNA analysis. The F_2 offspring were checked for the recovery of parental AFLP alleles that were generated as described in Pannebakker *et al.* (2004a) with the primer combinations *Mse*-CA/*Eco*-ACA and *Mse*-CA/*Eco*-AGG.

If genomic incompatibilities exist between the two modes of reproduction, they will be detectable by an unequal recovery of parental alleles in the F_2 recombinant males (cf. Gadau *et al.*, 1999). When incompatibilities are absent, equal proportions (1/2) of alleles from both reproductive modes are expected in the F_2 males, due to meiosis in the hybrid F_1 females. We tested for equal recovery of the parental alleles using a binomial test (Zar, 1996; Schork & Remington, 2000).

Results

Courtship and copulation experiments

Courtship behaviour

Courtship behaviour was described for matings between virgin males and females from the uninfected population DC-E00. Typical courtship behaviour is illustrated in Fig. 1, while the sequence used for recording of male courtship behaviour is illustrated in Fig. 2. The sequence of courtship behaviour in *L. clavipes* resembles that of other Eucoline parasitoids [i.e. *L. heterotoma* = *Pseudeucoila bochei* (van den Assem, 1969) and *Aganaspis pelleranoi* (Ovruski & Aluja, 2002)].

Male courtship behaviour differentiation

The courtship behaviour of the ACI males and the males from the arrhenotokous population is summarized in

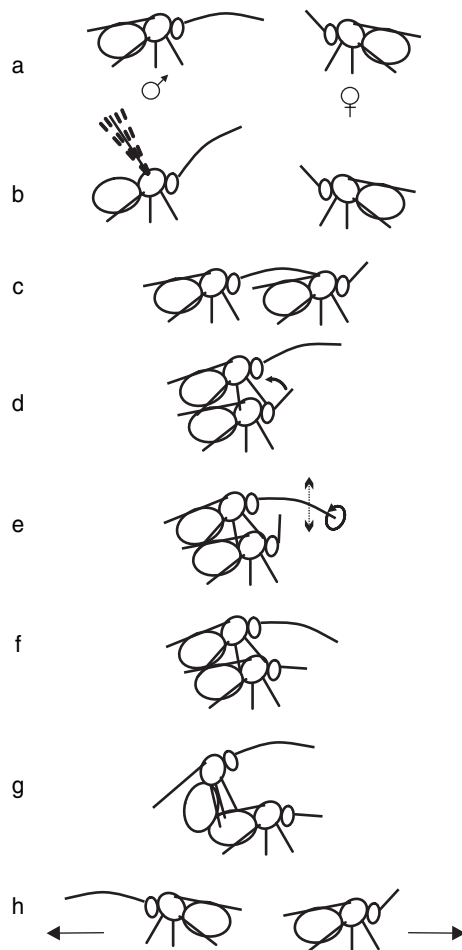


Fig. 1 Schematic representation of male courtship behaviour in uninfected *Leptopilina clavipes*. (a) Male introduced to mating arena. (b) Following detection of the female, the male starts vigorous wing fanning until genital contact is made. (c) While wing fanning, the male follows the female and makes antennal contact with the female's dorsal gaster or thorax upon which receptive females remain motionless. (d) Male mounts female until his head is over that of the female after which the female raises her antennae in an upward position. (e) Male starts antennation, consisting of male–female antennal contact (antennal sweeps) and wing fanning bouts. Antennal sweeps are characterized by forward extension of the antennae, which are both then raised and lowered simultaneously in small circular movements. (f) After a number of antennal sweeps, a receptive female moves her antennae from a straight upward to a downward position after which she opens up her abdomen. (g) Upon this signal, the male stops the antennal sweeps and wing fanning and begins copulation. During copulation, the female remains arrested, while the male slowly moves his antennae up and down. (h) Copulation is usually terminated by the female by pushing the male with her hind legs and removing him from her back.

Table 1. The transition to wingfan that indicates detection of the resident virgin female shows significant differences between the lines (Table 1). This difference,

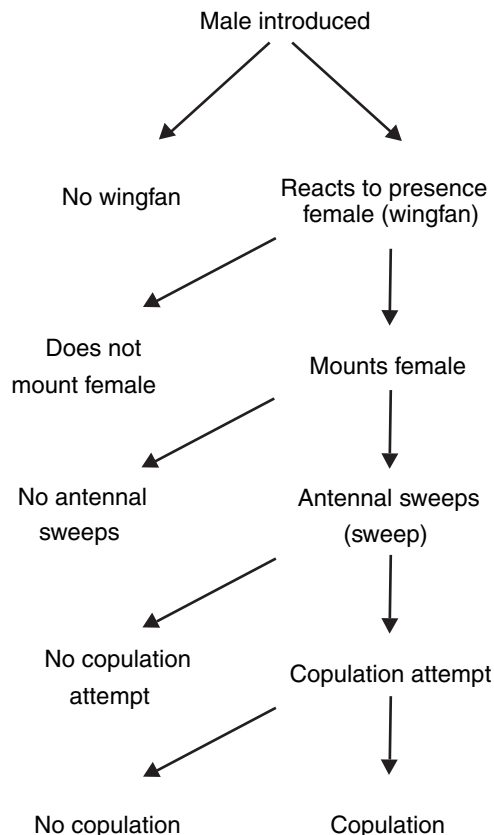


Fig. 2 Sequence of male courtship behaviour in *Leptopilina clavipes* used in the behavioural recordings.

however, is caused solely by the difference in transition to wingfan of the thelytokous line GBW-NL00 with the arrhenotokous and, all the other ACI males ($F_{8,102} = 3.47$, $P < 0.001$). No differences in further transitions towards copulation were found, although considerable variation exists in the proportion of copulations that result in successful fertilization.

The quantitative behavioural traits show high variability between the different lines. However, no consistent differences were found in male courtship behaviour between males from the arrhenotokous population and ACI males from the thelytokous populations.

Sex ratio experiments

The sex ratios (proportion of male offspring) produced by ACI males from the thelytokous lines are significantly higher ($F_{1,8.65} = 23.06$, $P < 0.05$, Tables 2 and 3) than those produced by the natural arrhenotokous males. No significant differences were found between arrhenotokous males from CCAP-E00 and DC-E00. Within the thelytokous males, however, the variance is higher than among the females (Tables 2 and 3). There is overall only a weakly significant effect of male line within the sex

Table 1 Transitions and quantitative data on male *Leptopilina clavipes* courtship behaviour from arrhenotokous (DC-E00) and thelytokous lines (BBH-NL00, DB17/9-NL99, DBK-NL00, GBW-NL00, HOD-NL00, KBH-NL00, NEU-F01, VOS-NL00).

Transitions					Quantitative data							
Line	N	Wingfan	Mount	Antennal sweeps	Copulation attempts	Copulation	Fertilization	Wingfan latency	Mounting latency	No. of antennal sweeps	Last mount-copulation duration	Copulation duration
DC-E00	24	0.96 ^a	0.70	1.00	0.94	0.93	0.64	85.3 ± 90.5	141.3 ± 135.5	19.6 ± 42.5	26.3 ± 11.8	66.9 ± 14.3
BBH-NL00	13	0.85 ^a	0.91	1.00	0.90	0.89	0.25	42.7 ± 52.0	175.9 ± 182.0	13.0 ± 11.8	25.3 ± 5.7	65.8 ± 14.9
DB17/9-NL99	13	0.85 ^a	0.82	0.89	1.00	0.75	0.83	132.4 ± 223.2	265.1 ± 243.1	37.4 ± 71.9	27.7 ± 17.2	114.8 ± 121.4
DBK-NL00	6	1.00 ^a	0.83	0.80	1.00	0.50	1.00	72.9 ± 69.3	143.3 ± 90.7	69.7 ± 127.8	47.9 ± 25.4	57.9 ± 2.2
GBW-NL00	11	0.27 ^b	0.67	1.00	1.00	0.00	–	101.8 ± 97.6	118.2 ± 144.2	10.7 ± 29.3	–	–
HOD-NL00	11	0.82 ^a	0.89	1.00	1.00	0.63	0.40	160.5 ± 250.1	214.2 ± 245.2	22.7 ± 26.3	41.8 ± 49.5	58.8 ± 17.3
KBH-NL00	13	0.85 ^a	0.91	1.00	0.90	0.78	0.86	63.9 ± 45.3	142.0 ± 89.1	15.5 ± 21.2	46.9 ± 41.0	56.1 ± 9.7
NEU-F01	11	1.00 ^a	1.00	1.00	1.00	0.82	0.67	11.6 ± 9.2	80.4 ± 60.2	22.5 ± 17.3	57.8 ± 48.5	90.4 ± 75.0
VOS-NL00	9	0.89 ^a	0.88	1.00	1.00	0.71	0.40	191.4 ± 326.0	152.1 ± 218.9	29.8 ± 50.3	31.8 ± 28.4	69.8 ± 20.9
		$F_{8,102} = 3.76$ $P = 6.7 \times 10^{-4}$	$F_{8,85} = 1.09$ $P = 0.38$	$F_{8,78} = 1.31$ $P = 0.23$	$F_{8,67} = 0.69$ $P = 0.70$	$F_{8,65} = 1.86$ $P = 0.07$	$F_{8,48} = 1.49$ $P = 0.19$	$F_{8,84} = 1.30$ $P = 0.26$	$F_{8,70} = 0.97$ $P = 0.47$	$F_{8,102} = 1.11$ $P = 0.36$	$F_{7,50} = 1.21$ $P = 0.32$	$F_{7,50} = 1.00$ $P = 0.44$

Durations and latencies are given in seconds ± SD. When statistical differences are present, different superscript letters indicate different groups.

ratios produced by thelytokous males, because of wide variance within the groups. It should be noted that although a difference in brood size was found between the lines ($F_{8,72} = 4.92$, $P < 0.001$, Table 2), this did not represent a difference between the reproductive modes ($t = -0.2474$, d.f. = 53.562, n.s., Table 2).

For most lines, the sex ratio of the first clutch was on average lower than that of the second clutch. A clear negative effect of the size of the first clutch on sex ratio was found ($F_{1,130} = 15.07$, $P < 0.001$, Table 3). No significant interaction between mode of reproduction and clutch was found, indicating that the patterns of the first vs. the second clutch is consistent for both arrhenotokous and thelytokous males. Brood size also had a negative effect on the sex ratio ($F_{1,134} = 6.72$, $P < 0.05$, Table 3).

Sperm use by infected females

Infected females mated readily with arrhenotokous males but did not produce hybrid offspring. Paternal markers were not incorporated into the genome of the offspring in any of the nine analysed matings (Table 4). Hence, infected females do not fertilize their eggs when mated to arrhenotokous males.

Recovery of parental alleles

The mean proportion of KBH-NL00 alleles in the F_2 recombinant males (0.542, SD = 0.164; Fig. 3) showed no significant deviation from that expected under equal recovery ($z = 0.705$, n.s.). Hence, there was no evidence of genomic incompatibilities between the two modes of reproduction.

Discussion

Sexual function of PI *Wolbachia*-infected *L. clavipes*

The experiments on sexual function of thelytokous *L. clavipes* showed that males produced by antibiotic treatment are able to mate successfully and sire offspring with arrhenotokous females. Although thelytokous females mate successfully with arrhenotokous males, they do not use the sperm to fertilize their eggs.

Courtship behaviour

Except for males from the GBW-NL00 line, ACI males from thelytokous *L. clavipes* lines are able to perform a complete courtship sequence, eventually resulting in successful copulation. This confirms results in other PI *Wolbachia*-infected species where male courtship behaviour of ACI males was studied (Pijls *et al.*, 1996; Arakaki *et al.*, 2000).

Besides the low transition to wingfan in GBW-NL00 males, no other differences in courtship were found between the thelytokous lines. Nevertheless, males from the arrhenotokous line and different thelytokous lines showed substantial variation in their behaviour, both within and between the lines. The variation within the

Population	Reproductive mode	Sex ratio (SE) first clutch	Sex ratio (SE) second clutch	Broodsize (SE)
CCAP-E00	A	0.158 (0.004)	0.286 (0.009)	138.00 (7.48) ^{ab}
DC-E00	A	0.157 (0.009)	0.280 (0.011)	119.25 (6.60) ^a
DB23/9-NL99	T	0.465 (0.061)	0.410 (0.035)	118.40 (7.22) ^{ab}
DBK-NL00	T	0.322 (0.030)	0.397 (0.022)	102.20 (12.98) ^a
GBW-NL00	T	0.775 (0.023)	0.808 (0.011)	110.83 (7.68) ^a
KBH-NL00	T	0.649 (0.020)	0.879 (0.010)	165.67 (7.27) ^b
VOSB-NL00	T	0.724 (0.018)	0.852 (0.009)	140.83 (14.50) ^{ab}
NEUVIC-F01	T	0.973 (0.006)	0.908 (0.020)	119.00 (12.75) ^{ab}
RENNES-F01	T	0.500 (0.030)	0.677 (0.019)	139.00 (9.60) ^{ab}

A, arrhenotokous; T, thelytokous. Standard error in parentheses. Different letters indicate statistical differences in broodsize at $P < 0.05$.

Effect	Estimate*	SE	d.f.	F	P	Variance estimate	CI
Fixed							
Arrhenotokous	-0.526	0.230	1	23.06	0.0011		
Thelytokous	1.547	0.466					
First clutch	-0.504	0.130	1	15.07	0.0002		
Second clutch							
Brood size	-0.006	0.002	1	6.72	0.0106		
Random							
Female/thelytokous						0.138	0.041–2.971
Male/thelytokous						0.985	0.384–5.946
Residual variance						7.279	5.790–9.469

*Back-transformed fixed effect parameter estimates to sex ratio, with brood size covariate fixed at the average over the total dataset: arrhenotokous, first clutch: 0.195; second clutch: 0.287; thelytokous, first clutch: 0.659; second clutch: 0.761.

Mother	Father	No. of females tested	No. of unique loci		Mean proportion recovered in offspring		No. offspring tested
			Maternal	Paternal	Maternal loci	Paternal loci	
BBH-NL00	Moll1-E00	2	10	2	1.00	0.00	10
			18	6	1.00	0.00	7
KBH-NL00	DC-E00	6	12	7	0.92	0.00	4
			15	5	1.00	0.00	10
			15	3	0.99	0.00	10
			19	4	0.99	0.00	10
			7	6	1.00	0.00	8
HOD-NL00	Moll1-E00	1	9	5	1.00	0.00	10
			9	5	0.64	0.00	10

arrhenotokous and thelytokous lines is most likely due to small age differences between the tested wasps, as age was not strictly controlled for.

Sex ratio

The sex ratios produced by males from thelytokous lines are higher than those produced by arrhenotokous males. Because *L. clavipes* has haplodiploid sex determination (i.e. females develop from fertilized and males from unfertilized eggs), a higher sex ratio implies a lower

fertilization success for the thelytokous males. As no difference in sex ratio was found between the two arrhenotokous lines, the effect is presumably due to the difference in reproductive mode rather than to the differences among populations.

For most lines, the sex ratio of the first clutch was lower than the sex ratio of the second clutch. Because females mated only once, the number of sperm in the spermatheca diminishes after ovipositing the first clutch which could have limited the production of female

Table 2 Reproductive mode, mean sex ratio of first and second clutch, and mean total broodsize produced by DC-E00 females after mating to males from different populations.

Table 3 Generalized linear mixed model for sex ratios. Note that the parameter estimates of fixed effects are given as logits and need to be transformed to obtain predicted mean sex ratios.

Table 4 Sperm utilization by *Wolbachia*-infected thelytokous *Leptopilina clavipes* females when mated with arrhenotokous males

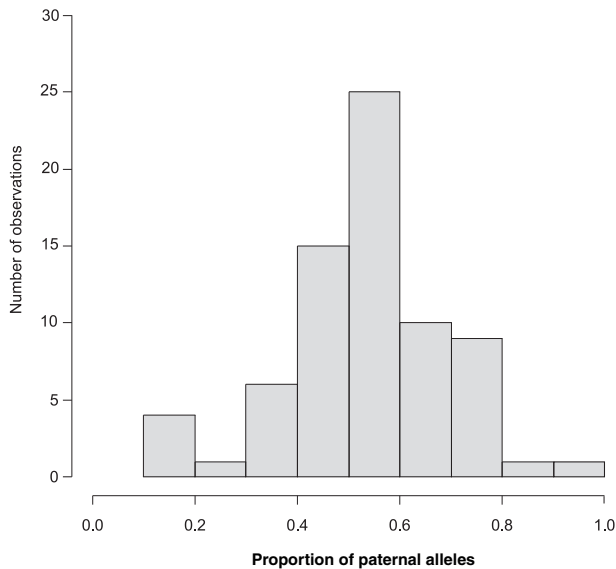


Fig. 3 Proportion of KBH alleles in each of the 72 hybrid F2 males resulting from a cross between a DC-E00 female and a KBH-NL00 male.

offspring in the second clutch (Godfray, 1994). Another possibility is low sperm viability, because of which many sperm could have been rendered nonfunctional between oviposition of the first and second clutch. Only NEUVIC-F01 and DB23/99-NL99 showed a lower second sex ratio.

Measured over all lines, brood size had a negative effect on sex ratio. The opposite effect might be expected when sperm is the limiting factor for sex ratio. However, according to the local mate competition (LMC) theory (Hamilton, 1967) a female should produce just enough males to fertilize her own daughters. If the LMC model is modified to include variation in brood size, it predicts large clutches to be relatively female biased (Werren, 1980), i.e. a negative effect of brood size on sex ratio.

In contrast to the observations in the courtship experiment, males from all the tested thelytokous lines mated and produced offspring, including males from GBW-NL00. The reasons for this difference are unclear, but may relate to differences in air pressure between the experiments. *Leptopilina* are known to adjust their behaviour to fluctuations in barometric pressure (Roitberg *et al.*, 1993) which we could not control for in our experiments. This is further supported by a futile attempt to start the sex ratio experiment in the winter of 2003. During this trial, courtship was only displayed by the arrhenotokous males (data not shown).

Thus far, *L. clavipes* is the only species in which a difference in fertilization success was found between males of arrhenotokous lines, and those induced from allopatric thelytokous populations. Males induced from

thelytokous *Trichogramma deion*, *T. pretiosum*, *Apoanagyrus diversicornis* and *Telonomus nawai* populations do not produce a higher sex ratio than their arrhenotokous conspecifics when mated to arrhenotokous females (Stouthamer *et al.*, 1990b; Pijls *et al.*, 1996; Arakaki *et al.*, 2000).

Sperm use

The AFLP analyses indicate that *Wolbachia*-infected *L. clavipes* females do not incorporate paternal genes into their offspring after mating to arrhenotokous males. Thus although thelytokous *L. clavipes* females readily mate with arrhenotokous males, they do not use the sperm to fertilize their eggs. Failed attempts to establish cured thelytokous lines (data not shown) support these results.

Failure to establish arrhenotokous lines from PI *Wolbachia*-infected lines has been reported for many species. For example, Arakaki *et al.* (2000) found no evidence of sperm use after mating by cured females from the parasitoid *Telonomus nawai*. In other species sperm transfer does not occur because females are not receptive to courting males [e.g. *Apoanagyrus diversicornis* (Pijls *et al.*, 1996) and *Muscidifurax uniraptor* (Gottlieb & Zchori-Fein, 2001)].

The pattern of sexually functional males and dysfunctional females found in *L. clavipes* is consistent with that observed in other parasitoids where completely infected and uninfected populations occur allopatrically (Pijls *et al.*, 1996; Arakaki *et al.*, 2000). Also in species where only infected populations were studied, matings between infected females and cured males do not result in fertilized eggs (Zchori-Fein *et al.*, 1992, 1994, 1995; De Barro & Hart, 2001; Gottlieb & Zchori-Fein, 2001; Weeks & Breeuwer, 2001). Only infected females from populations where infected and uninfected females co-exist fertilize their eggs after mating with cured thelytokous or arrhenotokous males. Thus far, these mixed populations are only known from several *Trichogramma* species and cured infected females from these populations proved to be fully functional (Stouthamer *et al.*, 1990a,b, 2001; Stouthamer & Luck, 1993; van Meer, 1999).

The pattern of nonfertilizing females and (partly) functional males is in line with the 'virginity-mutant' hypothesis of Huigens & Stouthamer (2003). According to this scenario, a virginity mutation in *L. clavipes* females was selected for in the initial stages of the infection. The virginity mutation is then fixed in an all-female population, where male sexual function is no longer actively maintained by selection and is prone to decay due to the random accumulation of mutations (Muller, 1949). However, mutation accumulation predicts that losses of unused functions should accumulate stochastically (Cooper & Lenski, 2000), and different male sexual functions should decay in different thelytokous lines.

We found a reduction in male fertilization capacity in thelytokous lines of *L. clavipes* from different geographic

origins. There are several explanations possible for this pattern. First, the allopatric modes of reproduction may have been separated long enough for incompatibilities to arise, which can be expressed as a reduction in hybrid (female) offspring. However, in the present study we found no evidence for genomic incompatibilities. The observed pattern of equal recovery of maternal and paternal markers is consistent with other intraspecific crosses in Hymenoptera (Hunt & Page, 1995; Antolin *et al.*, 1996; Laurent *et al.*, 1998), but not with interspecific crosses (Gadau *et al.*, 1999). In addition, the genetic distance between the two modes of reproduction is low when compared with closely related outgroups (Pannebakker *et al.*, 2004c).

A second explanation is that the infected *L. clavipes* lines originate from the same infection and hence show the same phenotypes. Genetic analysis, however, showed that there are at least two major clonal genotypes present in north-western Europe (Pannebakker *et al.*, 2004c) which were both used in this study (DB/23/9-NL99 & VOS-NL00 vs. DBK-NL00, GBW-NL00, KBH-NL00, NEUVIC-F01 & RENNES-F01). Hence, the observed similarity in phenotypes between the lines is not expected based on their genotypic diversity.

A third explanation for the similar pattern in male fertility among different thelytokous lines is that it is the result of indirect selection due to antagonistic pleiotropy. This may then lead to the loss of sexual function in males due to selection on genetically linked traits that are adaptive in the parthenogenetic strains. When assumed that selection pressures for these traits are similar in different populations, the same sexual trait is expected to decline in different populations (cf. Cooper & Lenski, 2000).

Under the 'virginity-mutant' hypothesis of Stouthamer & Huigens (2003), strong selection on female virginity is expected in populations in the early stages of the infection. The data on *L. clavipes* show that females from infected populations do not fertilize their eggs after mating, while males from all infected populations examined show a reduced fertility. This pattern could indicate a role for antagonistic pleiotropy between the nonfertilizing mutation in females and the reduction in fertility in males. Linkage analysis in the KBH-NL00 strain identified a single quantitative trait locus (QTL) of large effect for the reduction in male fertility (Pannebakker *et al.*, 2004a), which suggests a key mutation in a single gene.

The role of antagonistic pleiotropy can be determined by comparing QTL involved in reduced male fertility in males induced from other infected populations than KBH-NL00. When the reduced sexual functionality is due to antagonistic pleiotropy, a QTL at the same genomic location is expected in males induced from other infected populations assuming similar selection pressures as predicted by the 'virginity-mutant' hypothesis. If the reduction in male fertility is the result of

antagonistic pleiotropy, the same gene or pathway should be responsible for nonfertilization in infected females. Examples of genes in other taxa where a single mutation would have such an effect include the gene coding for the sperm-egg attachment mediating protein binding in sea urchins (Palumbi, 1999), and various male-female-sterile (*mfs*) genes in *Drosophila* (i.e. *mfs(1)6E*, *mfs(2)350*, *mfs(3)73A* and *mfs(3)G*, Gelbart *et al.*, 2003) that have different functions in male and female reproduction (Fukunaga, 1980; Lopez *et al.*, 2001). It should be noted however, that linkage disequilibrium between the genes coding for nonfertilization in females and the reduced males fertility in males creates a similar pattern.

In conclusion, we found evidence for the existence of a single reproductive barrier between allopatric arrhenotokous and thelytokous *L. clavipes* populations. Females from *Wolbachia*-infected populations do not use sperm after mating to arrhenotokous males. Males induced from infected populations show normal courtship behaviour and are capable of fertilizing arrhenotokous females; however, with a lower fertilization success than arrhenotokous males.

Although the lack of genomic incompatibilities and a low genetic distance indicates the two allopatric modes of reproduction belong to a single species, the reproductive barrier between them may be a first step in the process of speciation. Due to sexual degradation, *Wolbachia*-infected populations can become 'locked' into thelytokous reproduction even if the infection is lost, eventually resulting in speciation between infected and uninfected populations (Werren, 1998; Bordenstein, 2003). In natural infected *L. clavipes* populations, presumably due to inefficient transmission of the *Wolbachia* bacteria, males are occasionally produced (Driessen *et al.*, 1990). Although these males can produce viable offspring when mated to arrhenotokous females, (unidirectional) gene flow between the two modes of reproduction is prevented by two additional barriers: a disjunct distribution and differences in phenology (Pannebakker *et al.*, 2004c). To unravel the evolutionary scenario, further detailed research into the ecological differences between the two modes of reproduction is needed, as well as an investigation of the *Wolbachia* infection history. Moreover, genetic characterization of the nonfertilization trait in infected females, as well as that of the reduced male fertility in different thelytokous lines can help to clarify the genetic mechanism and evolutionary history involved in the loss of sexual function in PI *Wolbachia*-infected species.

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